Process for determining atrial natriuretic peptide (ANP)

The subject invention relates to a process for determining atrial natriuretic peptide (ANP) and furthermore to in vitro diagnostic utilization of specific polyclonal antisera to the 98 amino acid N-terminal fragment (proANP 1-98) of human pro-atrial natriuretic peptide (126 amino acids) and its analogs in the field of veterinary medicine.

Clinical importance of natriuretic peptides

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) belong to a family of hormones which are secreted from the atrium, the ventricle and the vascular endothelial cells (1-3). ANP is stored in the myocytes in the form of a prohormone of 126 amino acids in length. Upon release the prohormone is cleaved equally into an N-terminal part of 98 amino acids of proANP (1-98) and the biologically active α-ANP (1-28) (3). The half life of proANP in plasma is markedly longer than that of α-ANP which has only a very short half life of 2.5 minutes (2), and the plasma concentration of proANP is up to 50 times higher than that of α-ANP (2-4). Since the circulating concentrations of immunoreactive proANP react with low sensitivity to rapid biological fluctuations of α-ANP, they reflect the total amount of secreted ANP.

The ability of natriuretic peptides to protect the organism from excess liquid and high blood pressure has previously been described in the literature (5). The biological, biochemical and pathophysiological role of natriuretic peptides has been summarized in review articles (6, 7).

Clinical value

The plasma concentration of proANP is elevated in patients with various forms of acquired hypertension, in particular if the blood pressure is very high as a result of left ventricular hypertrophy. After heart failure, the plasma concentration of proANP increases relative to the extent of damage to the heart. After acute myocardial infarction, the concentrations of all natriuretic peptides increase rapidly.

In all of these pathophysiological conditions mentioned, the circulating concentrations of natriuretic peptides are at an elevated level. This is a protective mechanism of the organism against angiostenosis and sodium retention. The plasma concentration of proANP was furthermore shown to be elevated in patients with heart defects relative to the severity of said heart defect and therefore substantially contributes to the prognosis. Of particular interest is the observation in many studies that the plasma concentration of proANP is significantly elevated even in asymptomatic patients with left ventricular dysfunction and therefore has an important clinical value as a noninvasive marker (9, 10, 11). Furthermore, a marked distinction of healthy control subjects and NYHA Class I patients has been shown (12). The development of methods of specific and exact measurement of proANP fragments is therefore of the highest medical interest.

Prior art

The only commercially available measuring method of determining proANP (1-98) is based on a radio-immunoassay (RIA) from BIOTOP, which has the usual disadvantages of competitive and radioactive assays, such as special rooms for radioactive work, waste disposal costs and often also poor reproducibility due

to high susceptibility to variations in the sample matrix. An alternative to the abovementioned RIA was presented by SHIONOGI & Co. LTD. (EP 0 721 105 A1). This involved producing monoclonal antibodies to positions 1-25 and 43-66 and using them in designing a radioactive or enzymatic sandwich immunoassay. However, the use of monoclonal antibodies requires expensive and methodically complex cell culture (production of mouse hybridomas or in vitro production) and obtaining the antibodies from the saliva of mice after intraperitoneal administration of said hybridomas, thereby limiting the achievable antibody yields and likewise greatly increasing the cost of the methods.

It was therefore necessary to develop methods which allow cost-effective production of relatively large amounts of antibodies (polyclonal) with a specificity equivalent to that of monoclonal techniques for utilization in a sandwich immunoassay.

According to the invention, use is made of polyclonal antibodies to epitopes of proANP (1-98) which are defined as immunogens 1, 2 and 3, and which specifically bind said immunogens and also recombinant proANP (1-98). This enables ANP to be detected easily and reliably and cardiac disorders to be diagnosed thereby in a reliable manner already at an early stage.

In order to readily find the antibodies, they are provided with a marker molecule, with preference being given to the marker molecule employed being a fluorescent substance, an enzyme or a dye.

Human or animal proANP (1-98) may be detected by contacting body fluid with a solution comprising any of the polyclonal antibodies to immunogen 1, 2 or 3 to form an antibody/proANP (1-98) complex followed by detecting the formation of said complex. The antibody/proANP (1-98) complex may be detected by way

of reaction with either of the other two antibodies in the form of a sandwich assay. The process of the invention is particularly reliable when a primary antibody to any of the immunogens is immobilized to a solid phase, the secondary antibody being employed in the reaction being one of the antibodies to either of the two remaining immunogens. The primary antibody may be immobilized to a microtiter plate, a membrane or solid particles.

A kit for carrying out the process of the invention may comprise the following components:

a) an immobilized primary antibody,

b) recombinant proANP (1-98) as a standard,

c) a polyclonal secondary antibody or a labeled detection antibody which binds specifically to said polyclonal secondary antibody.

The invention thus includes the selection of suitable partial sequences of the N-terminal fragment as immunogens which have been optimized with respect to their antigenicity by means of numerical methods and which, at the same time, have minimal cross reactions to other physiologically circulating N-terminal fragments (proANP (1-30), proANP (31-67)). Furthermore the development of a sandwich immunoassay for proANP (1-98) (analyte) using polyclonal antibodies to substructures of the analyte, which antibodies have been purified by immunoaffinity chromatography. The method of measuring the analyte includes the following steps:

Incubation of the sample solution to be examined with a polyclonal antibody to a substructure of the analyte and a labeled polyclonal secondary antibody to another substructure of the analyte and detection of the antigen-antibody complex formed.

In addition to the use of polyclonal antisera to partial sequences of proANP (1-98) and the in vitro diagnostic utilization thereof and also selection of suitable partial sequences of the N-terminal fragment as immunogens, the present subject matter also relates to the chemical synthesis of said immunogens and to the immunization of carrier animals (preferably sheep). The purification of the crude sera by immunoaffinity chromatography, conjugation of the antibodies obtained with a marker molecule (e.g. enzymes, biotin, colloidal gold, fluorescent or luminescent substances and radioisotopes) and assaying of the most suitable antibody combinations for detecting proANP (1-98) in the form of sandwich immunoassays were also carried out. The latter may be carried out in the following embodiments:

The polyclonal primary serum contains antibodies to epitopes of the partial sequence 8-27 and the secondary serum contains antibodies to epitopes of the partial sequence 79-98 or 31-67. Detection comprises detecting the resulting antigen-antibody complex.

The polyclonal primary serum contains antibodies to epitopes of the partial sequence 79-98 and the secondary serum contains antibodies to epitopes of the partial sequence 8-27 or 31-67. Detection comprises detecting the resulting antigen-antibody complex.

The polyclonal primary serum contains antibodies to epitopes of the partial sequence 31-67 and the secondary serum contains antibodies to epitopes of the partial sequence 8-27 or 79-98. Detection comprises detecting the resulting antigen-antibody complex.

In another embodiment, the antibodies of the particular secondary serum are labeled with an enzyme, biotin, colloidal gold, a fluorescent substance or luminescent substances or radioisotopes.

Accordingly, the present invention provides advantageously (1) a method of producing polyclonal antisera to proANP (1-98) with a specificity equivalent to monoclonal antibodies, (2) a sandwich immunoassay for biologically inactive proANP (1-98) and (3) polyclonal antisera to biologically inactive proANP (1-98) for use in histology and (4) an immunoassay kit for biologically inactive proANP (1-98), which contains said antisera.

Figure 1 depicts a typical standard curve of a sandwich ELISA for proANP (1-98)

Figure 2 depicts determination of proANP (1-98) concentrations in the blood of samples of patients with a different degree of cardiac disorder (NYHA I-IV)

Table 1 depicts the crossreactivity of the proANP (1-98) ELISA to other N-terminal natriuretic peptide fragments.

Selection and production of immunogens - immunization

The required high specificity and avidity of the desired antisera can be achieved only by appropriate selection of the immunogens used for immunization of carrier animals. The problems to be solved are:

a) the choice of a sufficient sequence distance between the peptides used for immunization in order to avoid crossreactivities to other naturally occurring proANP fragments,

b) to find the most suitable sequence for optimal immune response and specificity,

c) to monitor as accurately as possible the immune response of the carrier animals in order to determine the most suitable time for a second immunization in order for the antisera produced to have optimal avidity.

Therefore numerical methods (software: PeptiSearch of CoshiSoft Arizona USA) which determination of antigenicity (algorithms by Jameson-Wolf and Welling) were employed in the analysis of the analyte (proANP (1-98)), in order to obtain polyclonal antisera of maximum avidity. The regions with highest antigenicity identified were the sequences 14-24 (DFKNLLDHLEE) and 79-95 (SSDRSALLKSKLRALLT).

Taking this as a starting point, the following synthetic immunogens were used for immunizing sheep at Guildhay Ltd., Walnut Tree Close, Guildford, Surrey GU1 4UG, ENGLAND on behalf of BIOMEDICA:

Immunogen 1: amino acid sequence 8-27, based on proANP (SEQ ID No. 1)
Immunogen 2: amino acid sequence 31-64, based on proANP (SEQ ID No. 2)
Immunogen 3: amino acid sequence 79-98, based on pro ANP (SEQ ID No. 3)

Sequence listing:

<110> Biomedica GmbH

<120> Polyclonal antisera for detecting proANP (1-98)

<140> AT A 1618/98
<141> 09.29.1999

<160> 3

<210> 1
<211> 20
<212> PRT
<213> Homo Sapiens

<400>

Ser	Asn	Ala	Asp	Leu	Met	Asp	Phe	Lys	Asn
1				5					10
Leu	Leu	Asp	His	Leu	Glu	Glu	Lys	Met	Pro
				15					20

;

<210> 2
<211> 34
<212> PRT
<213> Homo Sapiens

<400>

Glu	Val	Val	Pro	Pro	Gln	Val	Leu	Ser	Glu
1				5					10
Pro	Asn	Glu	Glu	Ala	Gly	Ala	Ala	Leu	Ser
				15					20
Pro	Leu	Pro	Glu	Val	Pro	Pro	Trp	Thr	Gly
				25					30
Glu	Val	Ser	Pro						

<210> 3
<211> 20
<212> PRT
<213> Homo Sapiens

<400>

Ser	Ser	Asp	Arg	Ser	Ala	Leu	Leu	Lys	Ser
1				5					10
Lys	Leu	Arg	Ala	Leu	Leu	Thr	Ala	Pro	Arg
				15					20

All peptides were prepared by organochemical protective group synthesis according to the prior art and coupled either N-terminally or C-terminally to thyreoglobulin as carrier protein. Coupling reagents which may be used are, for example, bifunctional compounds such as 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), also sulfo-MBS, sulfo-SMCC or the like.

Said immunogens were used to subject sheep to a primary immunization by administering the peptide in a mixture with complete Freund's adjuvant. The immunoresponse was investigated by means of an antibody capture ELISA in which microtiter plates coated with the carrier peptide used for immunization were used. Serial dilutions of a serum sample freshly obtained from the sheep were incubated using said microtiter plates and specific binding of the antibodies was quantified using an anti-sheep peroxidase -IgG conjugate.

The antibody titer of the sheep was checked monthly, with appropriate second immunizations being carried out within in each case optimal intervals when the titer had decreased.

Obtaining the polyclonal sera - immunoaffinity chromatography

The crude sera obtained from the sheep were purified by affinity chromatography via HiTrap minicolumns (PHARMACIA, Sweden). About 0.5 mg of the appropriate peptide used for immunization was bound to said columns according to the protocol supplied by Pharmacia. After filtering the crude sera through a 0.45 µm Millex filter (MILLIPORE, USA), 10-20 ml of antiserum 1+2 (v/v) were diluted with 50 mM borate buffer pH 7 and applied to the column with a flow rate of 0.5 ml/min at room temperature. The specifically bound antisera were eluted with 0.1M citrate buffer pH 1.7 at a flow rate of 1 ml/min. Elution was monitored by means of a 280 nm UV detector and fractions of in each case 0.5 ml antiserum were collected on in each case 0.5 ml of initially provided 0.5M borate buffer pH 10, in order to achieve immediate neutralization of the eluate. The IgG concentration of the eluate was determined using a commercial protein detection method (µBCA by PIERCE, NL).

Recombinant proANP (1-98), expressed in E. coli, from the Institute for Microbiology of the University of Vienna was used as standard material.

Examples

The described polyclonal antisera to the immunogens 1-3 of the present invention may be employed in all known immunoassay variants such as

a) enzyme linked immunosorbent assays (ELISAs), including automated hybrid methods (e.g. using

polystyrene or latex beads) in microtiter plates or on membranes

b) fluorescent immunoassays (FIA)

c) various test strip methods based on dry chemistry

d) histological detection on different tissue preparations.

Some embodiments will be described by examples below:

Example 1

Sandwich ELISA for proANP (1-98)

Aliquots of the purified sera to immunogens 1, 2 and 3 were labeled with biotin using biotinamidocaproate N-hydroxysuccinimide ester or the like according to standard methods (8). Recombinant proANP (1-98), expressed in E. coli, from the Institute for Microbiology of the University of Vienna was used as standard material for the immunoassay.

The following protocol represents a typical assay procedure:

Microtiter plates (Nunc Maxisorp High Binding, NUNC, Denmark) are coated with 200 µl of antiserum dilution (primary serum) to, for example, immunogen 1 at 4°C overnight. Unspecific binding sites are blocked and standard or sample is mixed with biotin-labeled antiserum to, for example, immunogen 3 in the well. After 2 h at 37°C, the wells are washed and streptavidin-peroxidase conjugate is added. After another hour of incubation at 37°C and another washing step, tetramethylbenzidine (TMB) is added, and finally color development which is proportional to the proANP (1-98) concentration in the sample is determined in a microtiter plate photometer.

Example 2

Direct fluorescent immunoassay for proANP (1-98)

In another embodiment of the invention, fluorescent dyes (fluorescein, rhodamine etc.) may be employed as markers for the antiserum to immunogen 3, for example. The assay may then be carried out as in example 1, with the addition of substrate not being necessary. It is furthermore possible to employ the fluorescently labeled antisera for histochemical studies concerning the distribution of proANP (1-98) in tissues (confocal laser microscopy, fluorescence microscopy etc.)

Example 3

Homogeneous immunoassay for proANP (1-98)

In another embodiment of the invention, the antibodies of the primary serum are bound to plastic particles (latex, polystyrene etc.) and added together with the labeled secondary antibody to the sample in a homogeneous solution. After a step of separation by filtration or centrifugation, the amount of secondary antibody bound is determined by way of a color reaction with a suitable enzyme substrate, using a conventional spectral photometer.

TABLE 1

Parameter	% Signal in proANP (1-98) -Sandwich ELISA
proANP (1-98)	100.00
proANP (1-30)	<1
proANP (31-67)	<3
proBNP (8-29)	<1
proBNP (32-57)	<1
proCNP (24-42)	<1
proCNP (53-73)	<1
proCNP (74-102)	<1
α-ANP (1-28)	<1

7. References

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2. Yandle T.G. et al. (1986), Life Sci. 38: 1827-1833
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11. Hall C. et al. (1994), Circulation 89: 1934-1942
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Claims:

1. A process for determining atrial natriuretic peptide (ANP), characterized in that polyclonal antibodies to epitopes of pro ANP (1-98) which are defined as immunogens 1, 2 and 3 (SEQ ID No. 1, 2 and 3) are employed, and which specifically bind said immunogens and also recombinant pro ANP (1-98).

2. The process as claimed in claim 1, characterized in that the antibodies are provided with a marker molecule.

3. The process as claimed in claim 2, characterized in that the marker molecule employed is a fluorescent substance, an enzyme or a dye.

4. The process as claimed in any of claims 1 to 3, characterized in that human or animal pro ANP (1-98) is detected by contacting body fluid with a solution comprising any of the polyclonal antibodies to immunogen 1, 2 or 3 to form an antibody/pro ANP (1-98) complex followed by detecting the formation of said complex.

5. The process as claimed in claim 4, characterized in that the antibody/pro ANP (1-98) complex is detected by way of reaction with either of the other two antibodies in the form of a sandwich assay.

6. The process as claimed in any of claims 1-5, characterized in that a primary antibody to any of the immunogens according to claim 1 is immobilized to a solid phase, the secondary antibody being employed in the reaction being one of the antibodies to either of the two remaining immunogens.

7. The process as claimed in claim 6, characterized in that the primary antibody is immobilized to a microtiter plate, a membrane or solid particles.

8. A kit for immunoassays for carrying out the process as claimed in any of claims 1 to 7, characterized in that it comprises the following components:

a) an immobilized primary antibody according to claim 6,

b) recombinant pro ANP (1-98) as a standard,

c) a polyclonal secondary antibody according to claim 6 or a labeled detection antibody which binds specifically to said polyclonal secondary antibody.

9. The use of the process as claimed in any of claims 4 to 7 for in vitro diagnosis and/or prognosis of cardiac disorders in human or veterinary medicine, with an increased pro ANP (1-98) concentration compared to healthy organisms indicating a cardiac disorder.

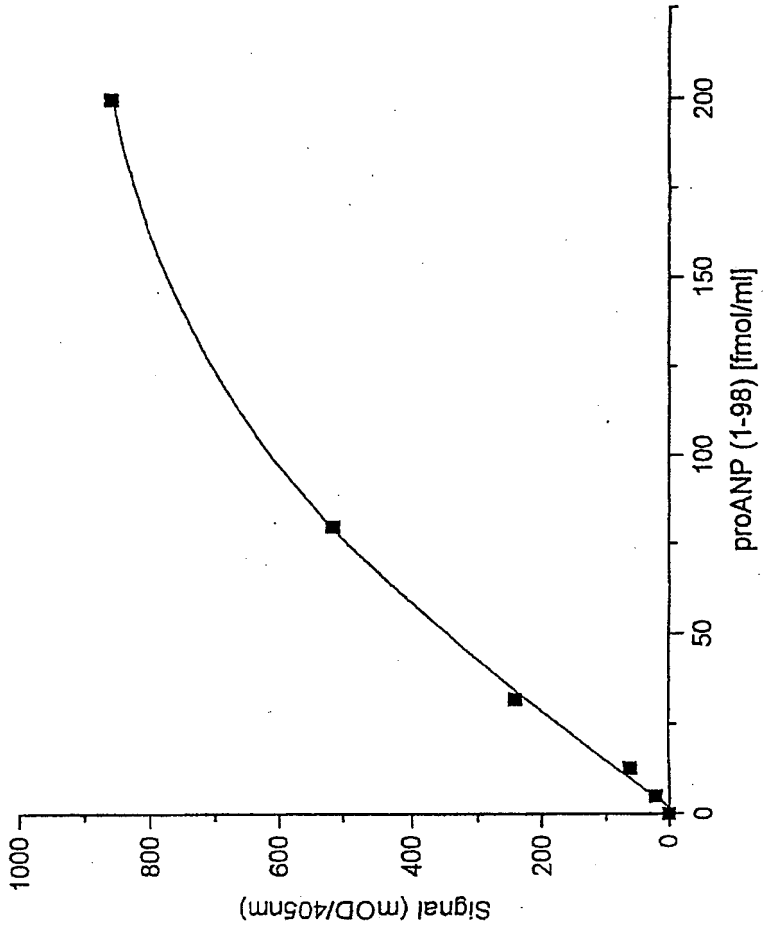
Signal (mOD/405nm)
1000
800
600
400
200
0
proANP (1-98) [fmol/ml]
0
50
100
150
200

Figure 1

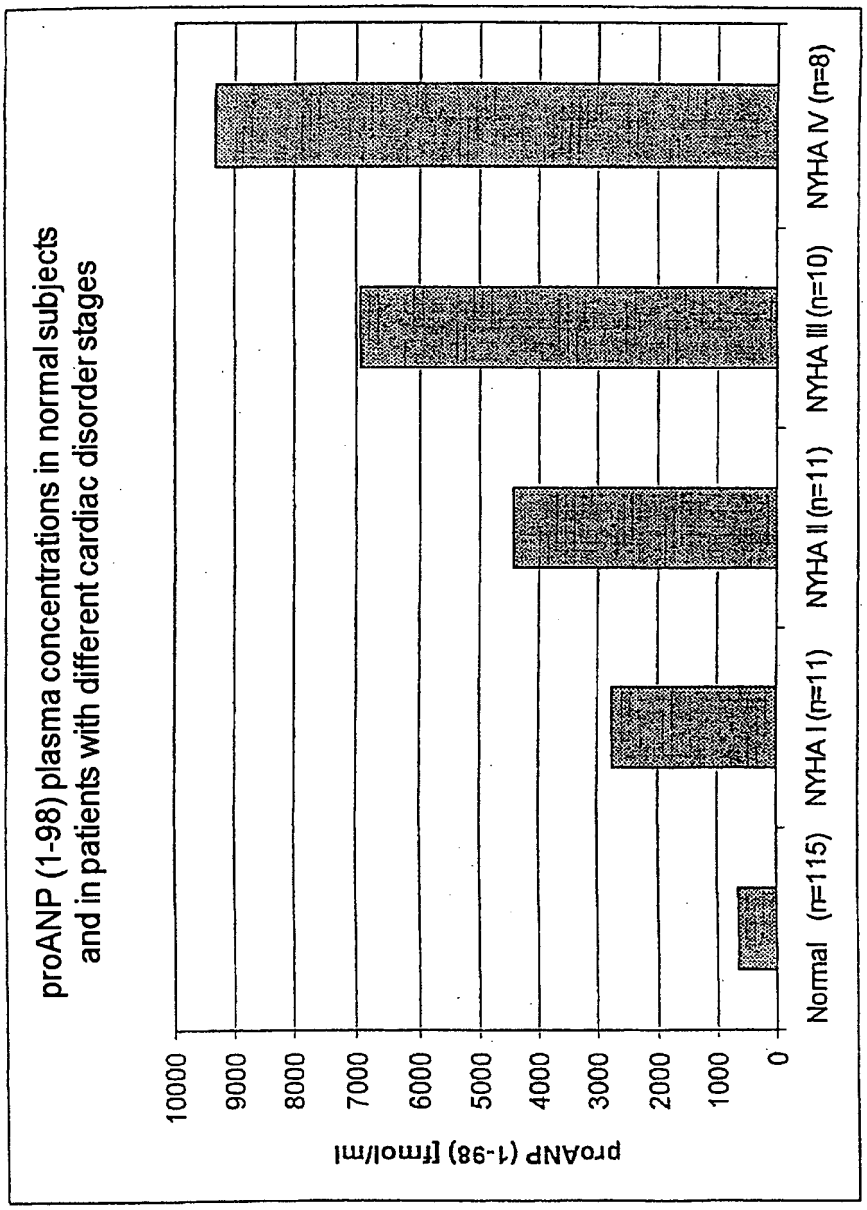
proANP (1-98) plasma concentrations in normal subjects and in patients with different cardiac disorder stages
proANP (1-98) [fmol/ml]
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Normal (n=115)
NYHA I (n=11)
NYHA II (n=11)
NYHA III (n=10)
NYHA IV (n=8)

Figure 2

Translator's Report/Comments

Your ref: N.88837B JHS Your order of (date): 22/08/2006

In translating the above text we have noted the following apparent errors/unclear passages:

Page/para/line*	Comment
Preferred title	"Process for determining atrial natriuretic peptide (ANP)"
3/11 + 13/10	First relative subclause ("die als...") relates to "Epitope" but second subclause ("und die diese...") should relate to "Antikörper", not to "Epitope".
5/28+29	"im Blut von Patientenproben" → "in Blutproben von Patienten"
6/9	"Sequenz-Abstandes" means evolutionary "distance"?
6/18+19	"welche...": incomplete subclause (verb missing)

* This identification refers to the source text. Please note that the first paragraph is taken to be, where relevant, the end portion of a paragraph starting on the preceding page. Where the paragraph is stated, the line number relates to the particular paragraph. Where no paragraph is stated, the line number refers to the page margin line number.

Translator's Report/Comments

Your ref: Your order of (date):

In translating the above text we have noted the following apparent errors/unclear passages which we have corrected or amended:

Page/para/line*	Comment

* This identification refers to the source text. Please note that the first paragraph is taken to be, where relevant, the end portion of a paragraph starting on the preceding page. Where the paragraph is stated, the line number relates to the particular paragraph. Where no paragraph is stated, the line number refers to the page margin line number.